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## **► To cite this version:**

Julien Cattel, Julien Martinez, F. Jiggins, L. Mouton, P. Gibert. Wolbachia -mediated protection against viruses in the invasive pest *Drosophila suzukii*. *Insect Molecular Biology*, 2016, 25 (5), pp.595-603. 10.1111/imb.12245 . hal-01916775

**HAL Id: hal-01916775**

**<https://hal.science/hal-01916775>**

Submitted on 7 Jan 2019

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***Wolbachia*-mediated protection against viruses in the invasive pest *Drosophila suzukii***

**J. Cattel<sup>\*§</sup>, J. Martinez<sup>†§</sup>, F. Jiggins<sup>†</sup>, L. Mouton<sup>\*</sup>, P. Gibert<sup>\*</sup>**

(<sup>§</sup>co-first authors)

<sup>\*</sup>Univ Lyon, Université Claude Bernard, CNRS, Laboratoire de Biométrie et Biologie  
Evolutive UMR CNRS 5558, F-69622 Villeurbanne, France; <sup>†</sup>Department of Genetics,  
University of Cambridge, Cambridge, United Kingdom

Running title: *Wolbachia* and antiviral protection in *D. suzukii*

Corresponding authors:

Julien Cattel ([juliencattel@gmail.com](mailto:juliencattel@gmail.com)) and Julien Martinez ([jtm35@cam.ac.uk](mailto:jtm35@cam.ac.uk))

## Abstract

The maternally inherited bacterium *Wolbachia* is well known for spreading in natural populations by manipulating the reproduction of its arthropod hosts, but can also have mutualist effects that increase host fitness. In mosquitoes and *Drosophila* some *Wolbachia* strains can lead to an increase in survival of virus-infected insects, and in most cases this is associated with reduced accumulation of the virus in host tissues. We have investigated if the *Wolbachia* strain *wSuz*, which naturally infects *Drosophila suzukii*, is able to confer protection against *Drosophila* C Virus (DCV) and Flock House Virus (FHV) in different host genetic backgrounds and we found that this strain can increase host survival upon infection with these two viruses. In some cases this effect was associated with lower viral titers suggesting that it is conferring resistance to the viruses rather than allowing the flies to tolerate infection. Our results indicate that, in *D. suzukii*, the antiviral protection provided by *Wolbachia* is not correlated to its density as found in other *Drosophila* species. This study demonstrates a phenotypic effect induced by *wSuz* on its native host which could explain its maintenance in natural populations of *D. suzukii*.

## Introduction

*Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae), the spotted-wing *Drosophila*, is an invasive species native to South East Asia (Kanzawa, 1936). It was originally described in Japan in 1916 and, within the last decade, it has been observed for the first time in California (Hauser, 2011), in Spain and in Italy (Calabria *et al.*, 2012) in 2008, and then quickly spread throughout North America and Europe (Cini *et al.*, 2012) and more recently in Brazil (Deprá *et al.*, 2014). In contrast to the vast majority of *Drosophila* species, *D. suzukii* is an agricultural pest because its serrated ovipositor allows it to lay eggs on healthy ripening fruits still attached to the plant (Mitsui *et al.*, 2006). Damage is caused by larvae feeding on the pulp inside the fruits and berries. As a consequence *D. suzukii* can have a severe economic impact, such as in the Western United States where it causes losses of up to US\$500 millions per year (Goodhue *et al.*, 2011). Because of its remarkable invasive success and impact on agricultural production, *D. suzukii* is currently subjected to intense research from both fundamental and applied perspectives.

Until now little was known about the symbiotic community of *D. suzukii*, despite maternally-inherited symbionts being common and important components of arthropod biology and ecology (Zchori-Fein & Bourtzis, 2011). Some studies revealed that *D. suzukii* naturally harbors *Wolbachia* (Cordaux *et al.*, 2008; Siozios *et al.*, 2013; Hamm *et al.*, 2014; Cattel *et al.*, 2016), which is the most common endosymbiont in arthropods with an estimation of 52% of arthropod species infected (Weinert *et al.*, 2015). Only one strain of *Wolbachia* has been identified in field populations of *D. suzukii* based on MLST markers, at least in North America and in Europe, which is closely related to *w*Ri (Siozios *et al.*, 2013; Hamm *et al.*, 2014; Cattel *et al.*, 2016). In many associations, the spread of *Wolbachia* in the host populations is achieved through their capacity to manipulate host reproduction either by

73 biasing the host's sex ratio towards the production of females or, more commonly, by  
74 impeding the reproduction of uninfected females through a sterility phenomenon called  
75 Cytoplasmic Incompatibility (CI) (Werren *et al.*, 2008). Theory predicts that the spread of CI-  
76 inducing *Wolbachia* in a population is under positive frequency-dependence and that their  
77 maintenance depends on their transmission efficiency and on the intensity of CI (Turelli &  
78 Hoffmann, 1995). *Wolbachia* can also successfully invade host populations by bringing direct  
79 fitness benefits to infected individuals such as increasing fecundity (Dobson *et al.*, 2002;  
80 Dobson *et al.*, 2004; Fry *et al.*, 2004; Weeks *et al.*, 2007; Unckless & Jaenike, 2012),  
81 longevity (Gavotte *et al.*, 2010; Brelsfoard & Dobson, 2011; Alexandrov *et al.*, 2007;  
82 Toivonen *et al.*, 2007) or provisioning nutrients (Brownlie & Johnson, 2009; Hosokawa *et al.*,  
83 2010; Unckless & Jaenike, 2012). In addition, *Wolbachia* can protect its host against viruses  
84 (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Osborne *et al.*, 2009; Bian *et al.*, 2010; Glaser *et*  
85 *al.*, 2010; Blagrove *et al.*, 2012). Such benefits could explain the presence in natural  
86 populations of *Wolbachia* strains that do not appear to rely on the reproductive manipulation  
87 to spread. For example, the strain wMel, which induces a very low level of CI (Hoffmann *et*  
88 *al.*, 1994; Hoffmann *et al.*, 1998), might be maintained in populations of *D. melanogaster*  
89 because of positive effects such as the protection it confers against several RNA viruses  
90 (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). Similarly, wAu, which naturally infects *D.*  
91 *simulans*, does not induce CI but confers strong protection against viruses (Osborne *et al.*,  
92 2009; Martinez *et al.*, 2014). This antiviral protection, which has been observed only in  
93 *Drosophila* and mosquitoes, has been shown to be highly variable according to the host  
94 species and the *Wolbachia* strain (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Osborne *et al.*,  
95 2009; Moreira *et al.*, 2009; Mousson *et al.*, 2010; Chrostek *et al.*, 2013; Chrostek *et al.*, 2014;  
96 Martinez *et al.*, 2014).

Previous studies found that the prevalence of *wSuz* is highly variable in populations of *D. suzukii* from North America (7 to 58%) and Europe (0 to 100%) (Hamm *et al.*, 2014; Cattel *et al.*, 2016) and, until now, there is no indication that this strain can induce strong reproductive manipulations in *D. suzukii* such as CI nor male killing (Hamm *et al.*, 2014; Cattel *et al.*, 2016). Moreover, in North American populations, it has been shown that *wSuz* is imperfectly vertically transmitted by wild-caught *D. suzukii* females, which would cause the bacterium to be lost from the population in the absence of any selection (Hamm *et al.*, 2014). All these results suggest that *wSuz* may bring a fitness advantage to *D. suzukii* but yet no effect has been found on fecundity, starvation tolerance or resistance to desiccation (Hamm *et al.*, 2014).

*wSuz* belongs to the supergroup A (Siozios *et al.*, 2013), which contains several *Wolbachia* strains known to induce antiviral protection (Martinez *et al.*, 2014). In the present study, we have thus tested whether *wSuz* can protect *D. suzukii* against viruses. Four host lines were compared, two from France, a country which was recently invaded by *D. suzukii*, and two from Japan, its native range (Cini *et al.*, 2012; Asplen *et al.*, 2015). Two RNA viruses were tested, *Drosophila C virus* (DCV; highly pathogenic *Drosophila* virus) and the Flock House virus (FHV; isolated from a beetle) (Scotti *et al.*, 1983; Huszar & Imler, 2008). We found that *wSuz* is able to protect *D. suzukii* against these two viruses but that the antiviral protection is very variable between the host lines. This beneficial effect could explain its maintenance in natural populations.

## Results

### *Wolbachia* protects *D. suzukii* against DCV infection

We measured the survival of Fr-CP (antibiotic-treated line) and Jp-OGH (introgressed line) flies uninfected or infected respectively with a French and Japanese *Wolbachia* isolate after inoculation with DCV (400 flies) or saline solution (Ringer, 400 flies) (Fig. 1A). In the mock-infected flies (Ringer's control treatment), the survival of *Wolbachia*-free and *Wolbachia*-infected individuals was not significantly different, indicating that there is no intrinsic effect of *Wolbachia* on the fly survival (Cox's mixed effect model; Main effect *Wolbachia*:  $\chi^2=0.92$ , d.f.=1,  $P=0.337$ ; Host genotype x *Wolbachia* interaction:  $\chi^2=1.57$ , d.f.=1,  $P=0.210$ ). However, the Fr-CP line had higher survival than the Jp-OGH line (Cox's mixed effect model;  $\chi^2=8.78$ , d.f.=1,  $P=0.003$ ).

We found that *Wolbachia* increased the survival of flies infected with DCV (Cox's mixed effect model:  $\chi^2=21.74$ , d.f.=2,  $P<0.001$ ; Fig. 1A) but the effect is significant for the Fr-CP line only (Cox's mixed effect model, Host genotype x *Wolbachia* interaction:  $\chi^2=4.1$ , d.f.=1,  $P=0.043$ ; Tukey test,  $P<0.001$  for Fr-CP and  $P=0.99$  for Jp-OGH). As Fr-CP and Jp-OGH lines differ in both the host and bacterial genotypes, either of these may be causing the difference.

The DCV titer was lower in *Wolbachia*-infected flies than in uninfected ones (Two-way ANOVA,  $F=15.22$ , d.f.=1,  $P<0.001$ ; Fig. 1B), and this effect of *Wolbachia* did not depend on the line (Two-way ANOVA, *Wolbachia* x host interaction:  $F=0.45$ , d.f.=1,  $P=0.509$ ; Fig. 1B).

#### *Wolbachia* effect on FHV infection

Given the difference in the degree to which *wSuz* increases the survival of *D. suzukii* after DCV infection between lines we then investigated the effect of *wSuz* on FHV infection in four genetic backgrounds: the effect of the French *Wolbachia* isolate, *wSuz-Fr*, in two French backgrounds Fr-CP and Fr-BE, and the effect of the Japanese isolate, *wSuz-Jp*, in two Japanese backgrounds Jp-OGH and Jp-YSG. A total of 800 flies were stabbed with FHV and 800 others with Ringer's solution (Fig. 2A). In the absence of viral infection neither *Wolbachia* nor the host genetic background affected survival (Ringer control treatment, Cox's mixed effect model, *Wolbachia* effect:  $\chi^2=1.83$ , d.f.=1,  $P=0.180$ ; host effect:  $\chi^2=1.43$ , d.f.=3,  $P=0.7$ ; *Wolbachia* x host interaction:  $\chi^2=1.22$ , d.f.=3,  $P=0.750$ ).

In FHV-infected flies, survival was significantly affected by the *Wolbachia* infection ( $\chi^2=31.88$ , d.f.=4,  $P<0.001$ ), the host genetic background ( $\chi^2=39.55$ , d.f.=6,  $P<0.001$ ) and we found a significant interaction between these two factors ( $\chi^2=14.99$ , d.f.=3,  $P=0.002$ ). Because we cannot exclude the possibility that the French and the Japanese lines are infected by a different *Wolbachia* isolate (*wSuz-Fr* and *wSuz-Jp* respectively), we also tested the *Wolbachia* and the host genetic background effects on infected flies' survival for the French and Japanese lines separately. The French lines survival was significantly affected by the *Wolbachia* infection ( $\chi^2=17.75$ , d.f.=2,  $P<0.001$ ), the host genetic background ( $\chi^2=34.14$ , d.f.=2,  $P<0.001$ ) but there was no significant interaction between these two factors ( $\chi^2=3.73$ , d.f.=1,  $P=0.053$ ). In the Japanese lines, the survival rate was affected by the *Wolbachia* infection ( $\chi^2=14.18$ , d.f.=2,  $P<0.001$ ), the host genetic background ( $\chi^2=10.54$ , d.f.=2,  $P=0.005$ ) and we detected a significant interaction between these two factors ( $\chi^2=8.41$ , d.f.=1,  $P=0.004$ ). By comparison with the uninfected lines, the *wSuz* infection significantly increased the survival of the Fr-BE and the Jp-YSG backgrounds (Tukey HSD,  $P=0.012$  and  $P<0.001$  respectively).



while it did not affect the survival of the Fr-CP and the Jp-OGH backgrounds (CP line,  $P=0.191$ ; OGH line,  $P=0.849$ ) (Fig. 2A).

As for DCV, we also measured FHV titers and we found a significant effect of both the *Wolbachia* infection status (Two-way ANOVA,  $F=5.04$ , d.f.=1,  $P=0.03$ ) and the host genetic background (Two-way ANOVA,  $F=98.88$ , d.f.=1  $P<0.001$ ) on the RNA copy number (Fig. 2B), with a significant interaction between these two factors (Two-way ANOVA,  $F=11.54$ , d.f.=1,  $P<0.001$ ). As for the survival data analysis, we tested the influence of the presence of *Wolbachia* and the host genetic background for the French and the Japanese lines separately. For the French lines the RNA copy number was affected by *Wolbachia* infection (Two-way ANOVA,  $F=4.32$ , d.f.=1,  $P=0.045$ ), the host genetic background (Two-way ANOVA,  $F=189.82$ , d.f.=1,  $P<0.001$ ) with a significant interaction between these two factors (Two-way ANOVA,  $F=21.01$ , d.f.=1,  $P<0.001$ ). For the Japanese lines, we also found a significant interaction between the *Wolbachia* infection and the host genetic background (Two-way ANOVA,  $F=13.18$ , d.f.=1  $P<0.001$ ), a significant effect of the host genetic background (Two-way ANOVA,  $F=88.80$ , d.f.=1,  $P<0.001$ ) but we did not detect a significant effect of the *Wolbachia* infection (Two-way ANOVA,  $F=1.05$ , d.f.=1,  $P=0.311$ ). More precisely, in the presence of *wSuz*, the RNA copy number significantly decreased (around 50% of reduction; Fig. 2B) in the Fr-BE and Jp-YSG backgrounds infected with *wSuz*-Fr and *wSuz*-Jp isolates respectively (Tukey HSD,  $P<0.001$  and  $P=0.039$  respectively), the two lines that exhibited a significant effect of *Wolbachia* on survival after FHV infection, and not in the two other lines (Tukey HSD test, Fr-CP line,  $P=0.665$ ; Jp-OGH line,  $P=0.478$ ).

### *Wolbachia* density

*Wolbachia* density is known to be a major determinant of antiviral protection, with higher densities being associated to higher levels of protection (Chrostek *et al.*, 2014; Martinez *et al.*, 2014). We therefore measured *wSuz* density in the four lines and found significant differences (One-way ANOVA,  $F=10.07$ ,  $d.f.=3$ ,  $P<0.001$ ; Fig. 3): the two Japanese's backgrounds (Jp-OGH and Jp-YSG) showed a higher density than the two French backgrounds (Fr-CP and Fr-BE), but there was no significant differences between the two French lines (both infected by *wSuz*-Fr; Tukey HSD,  $P=0.991$ ) and between the two Japanese lines (that both harbor the Japan *Wolbachia* isolate ; Tukey HSD,  $P=0.062$ ).

### Discussion

We have found that *wSuz* can protect its host against RNA viruses. In certain lines individuals infected with *wSuz* had higher survival and lower viral titers after infection with DCV and FHV. It is known since 2008 that *Wolbachia* can protect *Drosophila* against RNA viruses (Hedges *et al.*, 2008; Teixeira *et al.*, 2008), but this is the first time that it is described in *D. suzukii*. In a recent study another direct fitness benefit of *Wolbachia* has been observed in an Italian population of *D. suzukii* since infected females have a higher fecundity than uninfected ones (Mazzetto *et al.*, 2015). These phenotypes can potentially explain the maintenance of *Wolbachia* strains in natural populations without reproductive manipulation (Fenton *et al.*, 2011), as it has been found in American and European populations of *D. suzukii* (Hamm *et al.*, 2014; Cattel *et al.*, 2016).

The variability of the *wSuz* prevalence could be the consequence of heterogeneity in virus-induced selection similarly to what was observed in the Pea Aphid *Acyrtosiphon pisum*. This species is protected against parasitoids by the symbiont *Hamiltonella defensa*,

which has variable prevalence among populations and is thought to be maintained by negative-frequency dependent selection depending on the parasitism pressure in the field (Oliver *et al.*, 2008). We found that *Wolbachia* mediated significant protection in *D. suzukii* (Fr-CP for DCV, Fr-BE and Jp-YSG for FHV) was associated with reduced viral titer. However, for DCV, the presence of *Wolbachia* correlates with a lower viral titer even when no effect on the flies' survival was detected (Jp-OGH line). Several studies showed that antiviral protection is generally explained by a phenomenon of resistance that reduces the accumulation of virus but, in some cases, no differences in viral titers were observed despite the protective effect (Teixeira *et al.*, 2008; Osborne *et al.*, 2009). In the latter case, it is possible that *Wolbachia* does not affect the replication of the virus but rather makes the host more tolerant to viral infection.

Experimental studies have shown that *Wolbachia*-mediated antiviral protection is a common phenomenon in *Drosophila* and mosquitoes (Bian *et al.*, 2010; Hedges *et al.*, 2008; Moreira *et al.*, 2009; Osborne *et al.*, 2009; Teixeira *et al.*, 2008; Chrostek *et al.*, 2013; Chrostek *et al.*, 2014; Martinez *et al.*, 2014) but is strongly dependent on the *Wolbachia* strain (Hedges *et al.*, 2008; Osborne *et al.*, 2009; Chrostek *et al.*, 2013; Chrostek *et al.*, 2014; Martinez *et al.*, 2014). For instance, Martinez *et al.*, 2014 showed that among 19 *Wolbachia* strains (originating from 16 *Drosophila* species) transferred into the same *D. simulans* genotype, only half of them induced protection against DCV and FHV. The effect of host genetics on protection is less well understood. However, the protective phenotype is affected by the host species. For example, the strain wInn protects its natural host *D. innubila* against FHV (Unckless & Jaenike, 2012) but has no effect in *D. simulans* (Martinez *et al.*, 2014). Here, we found that the level of antiviral protection varied among the lines we used. This difference was most dramatic in the DCV experiment, where we found large increases in the

survival of the French line but not the Japanese line. This difference could be caused by genetic differences between the *Wolbachia* isolates, the flies or both. In the FHV experiment we were able to compare the same *Wolbachia* isolates in two host genetic backgrounds. We found a host background effect for both the Japanese and the French lines suggesting that host factors may affect the expression of the *Wolbachia*-mediated protection. However, we would caution that this needs further confirmation as we only have a single replicate line of each *Wolbachia* isolate in each genetic background, so we cannot rule out other possible differences (e.g. gut microbiota, or uncontrolled differences in the genetic background). *Wolbachia* density is known to influence the level of protection (Osborne *et al.*, 2009; Osborne *et al.*, 2012; Chrostek *et al.*, 2013; Chrostek *et al.*, 2014; Martinez *et al.*, 2014). However, we didn't find any clear association between the level of protection and the density of *Wolbachia*. The variation in antiviral protection could also be influenced by tissue tropism of *Wolbachia* since Osborne *et al.*, 2012 highlighted that tissue tropism can partly explain variations in the level of protection. Therefore it is possible that, in the *D. suzukii* lines used in our study, the tissue tropism of *Wolbachia* was different despite showing very similar density at the whole fly level.

The importance of antiviral protection in natural populations of *D. suzukii* is unknown. It has been estimated that *Wolbachia* would need to generate a fitness benefit of 20% to be maintained in populations (Hamm *et al.*, 2014). To achieve this RNA viruses would need to be causing significant harm to the flies in nature and *Wolbachia* would need to be mitigating much of this harm. The effects of the presence of *Wolbachia* on viral titer and survival that we observed were mostly smaller than in many previous studies (Hedges *et al.*, 2008; Teixeira *et al.*, 2008; Chrostek *et al.*, 2013; Chrostek *et al.*, 2014; Martinez *et al.*, 2014). However, it is not possible to extrapolate this to effects in nature without further work.

## Experimental procedures

### *D. suzukii* lines and rearing

In this study, four lines of *D. suzukii* were used, two originating from France and two from Japan. The French lines were collected in Compiègne (named Fr-CP) and in Bellegarde (named Fr-BE) in 2011 and 2012 respectively and reared in large populations. The Japanese lines have been obtained from the Ehime-fly stock center in 2011: they were sampled in Yamagata (named Jp-YSG) (I#E-15016 YSG-11) and Tokyo (named Jp-OGH) (#E-15014OGH06-03) in 2006. These lines have been chosen because two are free of *Wolbachia* (Fr-BE and Jp-OGH) and the two others (Fr-CP and Jp-YSG) are 100% infected with *Wolbachia* (see below for diagnostic PCR test). The flies were reared on a cornmeal diet (agar: 1%, dextrose: 8.75%, maize: 8.75%, yeast: 2%, nipagin: 3%) and maintained in an incubator at constant temperature (22°C) and humidity (70%) with a 12-hours light/dark cycle. An MLST analysis performed on 6 genes (*ftsZ*, *fbpA*, *hcpA*, *coxA*, *gatB* and *wsp*) revealed the *Wolbachia* isolates from Fr-CP and Jp-YSG lines to be the same sequence type with 100% identity between the sequences. The sequences obtained in the present study are recorded in Genbank as KS308222-7.

### *Control of host genetic background and infection status*

We used two different methods to obtain *Wolbachia*-infected and *Wolbachia*-free lines with similar genetic backgrounds: antibiotic treatments of the infected lines and introgression of *Wolbachia* into uninfected lines by back-crossing.

Antibiotic treatments were performed for 3 generations in Fr-CP and Jp-YSG lines. At each generation larvae were fed on medium with 0.25 mg.mL<sup>-1</sup> tetracycline. After 3 generations, 10 isofemale lines were established from treated females and the presence of

*Wolbachia* was checked by PCR as described below in mothers and then for 3 generations more. Only one isofemale line was retained for each nuclear background (Fr-CP and Jp-YSG) and maintained for 12 generations before the experiments. The absence of *Wolbachia* in these lines was confirmed by real-time quantitative PCR (see below). Using this approach, we obtained infected and cured lines with the same genetic background, Fr-CP or Jp-YSG.

To obtain infected and uninfected individuals with the same Fr-BE or Jp-OGH genetic backgrounds, back-crosses were done for 8 generations. Two males from the uninfected line (Fr-BE or Jp-OGH) were mated with single virgin females from the infected lines from the same country, *i.e.* Fr-CP and Jp-YSG respectively. Backcrossing was performed for a total of 8 generations which lead to an introgression of around 99.6% of the nuclear background assuming no selection on the nuclear genome. However, compared with the use of antibiotics treatments, lines obtained with this method have different mitochondrial backgrounds. These two lines were maintained for 15 generations before the experiments. The *Wolbachia* infection status of each line was verified by PCR just before the viral infection experiment.

#### *Viral isolates*

Two viruses, *Drosophila C virus* (DCV) and *Flock House virus* (FHV), were used in this study. DCV is a highly pathogenic *Drosophila* virus, which belongs to the family *Dicistroviridae* (Huszar & Imler, 2008); FHV, which belongs to the *Nodaviridae* family, is not a natural pathogen of *Drosophila* species and was initially isolated from a beetle (Scotti *et al.*, 1983). Viruses were produced and titrated as described by Martinez *et al.*, 2014. DCV was produced and titrated in Schneider's Line 2 cells (SL-2) and FHV was titrated in Schneider *Drosophila* Line 2 cells (DL2) (<https://dgrc.bio.indiana.edu/cells/Catalog>). For each infection

assay, one viral aliquot was defrosted just before the infection and diluted in Ringer's solution (Sullivan *et al.*, 2000) to reach a viral concentration of  $5 \times 10^8 \text{ mL}^{-1}$  TCID<sub>50</sub> for DCV and  $3.6 \times 10^{10} \text{ mL}^{-1}$  TCID<sub>50</sub> for FHV.

### *Survival assay*

In order to test for a potential protective effect of *wSuz*, we measured the survival of flies after infection with DCV, FHV or mock infection with Ringer's solution. To infect flies, a 0.1 mm diameter anodized steel needle (26002-15, Fine Science Tools, CA, USA) was bent, 0.25 mm from the end, dipped in viral solution and the bent part of the needle pricked into the pleural suture on the thorax of flies (Longdon *et al.*, 2013). For DCV, we followed the survival of *Wolbachia*-free or *Wolbachia*-infected flies of the Fr-CP and Jp-OGH lines only. Since, in that first experiment, we observed variation depending on the geographical origin of the flies, we performed the second experiment with FHV using the four genetic backgrounds (Fr-CP, Fr-BE, Jp-OGH and Jp-YSG). Survival of Ringer's controls was followed in parallel for these two experiments.

For each line 3 days-old females were collected. After being anaesthetized with CO<sub>2</sub>, they were inoculated with DCV, FHV or Ringer's solution by stabbing flies. Groups of 20 stabbed flies were immediately placed into a vial of fly cornmeal medium and stored at 22°C. Flies were transferred into fresh vials of food every 3 days and the number of dead flies was recorded every day. The survival assay was replicated 5 times on independent cohorts of flies across multiple days, corresponding to a total of 100 flies for each *Wolbachia* infection status and virus infection treatment.

*Diagnostic polymerase chain reaction (PCR)*

The *Wolbachia* infection status of individuals was verified by PCR for each line just before performing the experiments. DNA was extracted on pools of 10 individuals (one pool per line) homogenized in 200µL of 5% w/v Chelex resin in water (Biorad) with 4µL of proteinase K (20mg.mL<sup>-1</sup>) and kept at 56°C for 3h. After 15min at 95°C, samples were centrifuged at 16000g for 4min and stored at -20°C. Presence of *Wolbachia* was checked by amplifying the *Wolbachia* Surface Protein (*wsp*) gene using the primers *wsp81F* and *wsp691R* (Braig *et al.*, 1998, Table S1). PCR reactions were performed in 25µL volumes containing 100µM dNTP, 200nM primers, 0.5IU DreamTaq® DNA polymerase (Eurobio) and 1µL of DNA template. Cycling conditions were 94°C (2min), 94°C (30sec), 52°C (30sec), 72°C (45sec), 72°C (10min) for 35 cycles. PCR products were visualized in 1% agarose gels.

*Real-time quantitative PCR (qPCR)*

The *Wolbachia* density, DCV and FHV RNA copy number were measured by real-time quantitative PCR (qPCR) on the Light Cycler<sup>TM</sup> system using primers listed in Table S1. To estimate *Wolbachia* density, 10 pools of ten 3 days-old virus-free females for each line were prepared and the DNA extracted using the Gentra Pure gene Tissue Kit (Qiagen). The *Wolbachia* density was measured by quantifying the copy number of the *Wolbachia* gene *ftsZ* relative to the host gene *Rpl32* using Sso Advanced Universal Probes Supermix (BioRad; 2min at 95°C followed by 40 cycles of 10sec at 95°C and 20sec at 60°C). The 10µL of multiplex reaction mix contained 400nM of *Rpl32* primers and 200nM of *ftsZ* primers, 5µL of SsoADVUniver Probes Supermix, 200nM of each probe and 2µL of DNA sample. The *Wolbachia* density was estimated by dividing the copy number of the *ftsZ* gene by the copy number of the *Rpl32* host gene. The antiviral protection was also examined by measuring the



RNA copy number after infection by both viruses. 3 days-old females were stabbed with DCV and FHV and frozen respectively 5 and 2 days after infection. After homogenization in TRIzol Reagent (Ambion), RNA was extracted from 10 pools of 10 flies for each experimental treatment using the RNA Easy Mini® kit following the manufacturer's instructions (Qiagen). Reverse-transcription was done using SuperScript® III First-Strand Synthesis System (Invitrogen) including a 30 min DNase digestion step at 37°C. The copy number of the viral RNA was compared to the control gene *Rpl32*. The qPCR reactions for DCV, FHV and *Rpl32* were done separately with the same conditions (30sec at 95°C followed by 40 cycles of 10sec at 95°C and 20sec at 60°C). The 10µL reaction mix contained 200nM of each primer, 5µL of SsoADV Univer SYBR Green Supermix, and 1µL of DNA sample. The RNA copy number and the *Wolbachia* density were estimated by calculating the ratio:  $\frac{E(\text{virus}/\text{Wolbachia})^{\Delta Ct}}{E(\text{host})^{\Delta Ct}}$  with  $\Delta Ct = Ct_{\text{flygene}} - Ct_{\text{virus}/\text{Wolbachia}}$  where  $E$  corresponds to the efficiency of the PCR reaction calculated from a dilution series for each set of primers ( $E = 2^{\frac{1}{\text{linear regression slope}}}$ ) and  $Ct$  to the cycle threshold (Pfaffl, 2001).

### *Statistical analysis*

Survival data were analyzed with a Cox's proportional hazards mixed-effect model using the coxme package in R (R Core team, 2013). The Cox's model estimates hazard ratios with the probability of a *Wolbachia*-infected fly dying at a given time-point divided by the probability of a *Wolbachia*-free fly dying. Flies that were alive at the end of the experiment were treated as censored data.

Survival data for DCV, FHV and their respective controls (Ringer) were analyzed separately. For each virus, two models were fitted to test a potential effect of the *Wolbachia*

infection and the genetic background on survival for the control treatment (Ringer) without virus or after infection with a virus. The first model allowed testing whether *wSuz* infection modifies survival independently of viral infection and indirectly confirm that the survival of virus-infected flies cannot be explained by an inherent effect of *Wolbachia* on survival. The effects of *Wolbachia*, host genetic background and their interaction were considered as fixed effects and the replicate vials as a random effect. When a significant interaction was detected, differences between *Wolbachia*-free and *Wolbachia*-infected flies within each host genetic background were analyzed using pairwise comparisons (Tukey's Honest Significance test) (R package multcomp).

Viral titers and *Wolbachia* density were analyzed on log2-transformed data. For viral titers, a two-way ANOVA allowed testing for the effect of *Wolbachia*, the host genetic background and their interaction. A one-way ANOVA was done to test for the influence of the host genetic background on *Wolbachia* density. Pairwise comparisons (Tukey's Honest Significance test) were also done if a global effect of *Wolbachia* was detected.

## Acknowledgements

This work was funded by CNRS (IFR41-UMR5558) and supported by ONEMA (APR Biodiversité-Ecophyto). J. Cattel is the recipient of a PhD studentship from the Rhône-Alpes region ("ARC Program" Grant).

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